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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Joseph M. Jilka
SERIAL NO: 10/086,062
FILED: February 28, 2002
TITLE: NOVEL PLANT PROMOTER SEQUENCES AND METHODS OF USE FOR SAME

ART UNIT: 1635
EXAMINER: Epps, J.

131 DECLARATION OF JOSEPH M. JILKA

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

I, Joseph M. Jilka hereby declare the following:

1. That I am the inventor for the above-identified patent application; that I conceived and reduced to practice in the United States the invention claimed in the above-identified patent application prior to the international publication date of March 23, 2000, of the cited PCT Application No. WO 00/15810 to Goldsbrough as evidenced by the enclosed notebook pages.

2. Attached Exhibit A is a copy of notebook records relating to this conception wherein construction of proposed versions of the ubiquitin variants show a no heat shock version. Also relating to this conception is Exhibit B which is a copy of a table listing the promoters made which show a no heat shock version. Attached Exhibit C are primers among which is the no heat shock version, version 4A, 4B.

3. That pursuant to this conception, I actually reduced to practice in the United States the invention claimed in the above-identified patent application prior to March 23, 2000, the international publication date of the cited Goldsbrough patent. Attached Exhibit D and E are copies of the notebook records of Kathy Beifuss, who worked under my direction and supervision, however, did not contribute materially to the above-identified invention, relating to the actual reduction to practice, wherein Exhibit D shows use the no heat shock

version in a mini-prep and Exhibit E shows use of the no heat shock version in sequencing. Additionally, attached Exhibits F and G relating to the actual reduction to practice is a copy of the notebook records of Chris Brooks and Elizabeth Wilfong, both who worked under my direction and supervision, however, did not contribute materially to the above-identified invention, showing the GUS reporter gene expression in corn seed using the Ubi promoter variant, GSC, the ubiquitin promoter having no heat shock elements. Wherein total soluble protein (1μg) was incubated in 100μl lysis buffer and the reaction initiated with 5mM 4-methylumbelliferyl β-D-glucuronide (MUG). The reaction was incubated for up to about 20 minutes at 37°C. At specific time points approximately 25μl of volume of the reaction mixture was transferred into a reading plate that had 175μl of Stop buffer in the well. The reaction plate was placed at 37°C until the next time point. Generally readings at 0, 15, 30, and 60 minutes were taken. Plates were read at 360nm excitation wavelength and 460 nm emission wavelength. GUS protein levels were then calculated by comparison to a standard curve of 1-100μM 4-methylumbelliferyl. Exhibit G shows results from a 10 minute reading. The dates of these records have been redacted, however, the acts of conception and reduction to practice occurred prior to March 23, 2000, the international publication date of the cited Goldsbrough patent.

4. That Exhibits, A, B, C, D, E, F, and G, which relate to the aforementioned conception and reduction to practice, correspond to the invention disclosed and claimed in the above-identified patent application.

5. The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Date: 7/17/02

Joseph M. Jilka

Construction of ubiquitin variants

Proposed versions

Version 1.0 ——→ A only
Version 2.0 ——→ B only
Version 3.0 ——→ A, B no overlap
Version 4.0 ——→ no A, B

Version 1.0

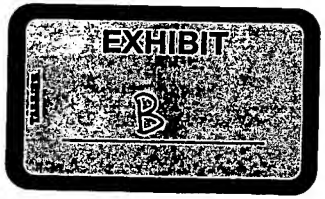
oligos A8333C11
A8333C10
diluted to 100 pmol/μl
50 μl each mixed
↓ 95°C 3 min
↓ slow cool
40°C

ligation 2 μl oligos (100 pmol)
2 μl 4-23C buffer
7 μl H2O
1.5 μl 10X buffer
1.5 μl 10 mM ATP
10 μl ligase
15 μl
↓ 16° 1 hr
↓ 65°C 15'

Sincerely yours,
Michele Barker

ProdiGene Promoters

Name	Description		Reporter	Test vector		Transform	Events
PGNpr1	maize polyubiquitin 1 (UBI1)		GUS	pPHP8904		Corn	GSA
			GUS-6xhis	pPGN7062		Corn	GSB
PGNpr2	maize globulin 1		GUS-6xhis	pPGN9075		Corn	
PGNpr3	maize 22 kD alpha-zein		GUS-6xhis	pPGN9071		Corn	PMD
PGNpr4	maize UBI1 no heat shock elements (HSE): UbiC		GUS-6xhis	pPGN7547		Corn	GSC
PGNpr5	maize UBI1 no 3' HSE: UbiD		GUS-6xhis	pPGN7565		Corn	GSD
PGNpr6	maize UBI1 no 5' HSE: UbiE		GUS-6xhis	pPGN7583		Corn	GSE
PGNpr7	maize UBI1 no HSE overlap: UbiF		GUS-6xhis	pPGN7600		Corn	GSF
PGNpr8	maize UBI1 replace HSE with 3x Pst1 seed specific element: UbiG		GUS-6xhis	pPGN8926		Corn	GSG
PGNpr9	teosinte polyubiquitin 1		GUS-6xhis	pPGN8984		Corn	GSI
PGNpr10	teosinte polyubiquitin 1a		GUS-6xhis	pPGN8985		Corn	GSM
PGNpr11	sorghum polyubiquitin 1		GUS-6xhis	pPGN8986		Corn	GSN
PGNpr12	maize glutathione-S-transferase 1 (GST1)		GUS-6xhis	pPGN8987		Corn	GSP
PGNpr13	synthetic promoter Rsyn0 with 35S enhancer 5' (tested with maize Adh-1 int		GUS-6xhis	pPGN9006		Corn	GSR
PGNpr14	synthetic promoter Rsyn7 with 35S enhancer 5' (tested with maize Adh-1 intr		GUS-6xhis	pPGN9007		Corn	GSS
PGNpr15	maize HRGP		GUS-6xhis	pPGN9016		Corn	SCA
PGNpr16	maize P promoter (tested with maize Adh-1 intron)		GUS-6xhis	pPGN9035		Corn	SCD
PGNpr17	modified version of Agro mannopine synthase (superMAS)		GUS	pPHP10336		Pea	GSH
PGNpr18	bean phaseolin		GUS-10xasp	pPGN9275		Pea	GSJ
			GUS-6xhis	pPGN5690		Pea	GSK
PGNpr19	maize UBI1 no 5' HSE with a little extended seq at 5' end (beyond pr6)		GUS-6xhis	pPGN9042		Corn	GST
PGNpr20	rice glutelin (1.3Kb of 5' sequence)		GUS-6xhis	pPGN9056		Corn	PMA
PGNpr21	rice glutelin 2.1Kb (2.3Kb of 5' sequence)		GUS-6xhis	pPGN9057		Corn	PMB
PGNpr22	rice globulin 26KDa		GUS-6xhis	pPGN9060		Corn	PMC
PGNpr23	maize globulin 2		GUS-6xhis	pPGN9076		Corn	



GIBCO BRL Custom Primers

Certificate of Analysis

Primer 1:

Primer Name: UBI HSP VER. 1A — Primer Number: A8333C10 (C10)

Researcher: — Primer Length: 66

Sequence (5' to 3'): PAG ACG GCA CGG CAT CTC TGT CGC TGC CTC CAC CGT TGG ACT TGC TCC GCT GTC GGC ATC CAG AAA T

Molecular Weight µg/µmole:	21299.2	µg per OD:	31.3	
Millimolar Extinction Coefficient:	678.6	nmoles per OD:	1.4	
Purity	**Desalted**	**OD's**	39.3	
Tm (1 M Na+)	96	**µg's***	1234	
Tm (50 mM Na+)	76	**nmoles**	57	~57
% GC	60	**Coupling Eff.**	99%	@ 1%

Notes:

Primer 2:

Primer Name: UBI HSP VER.1B — Primer Number: A8333C11 (C11)

Researcher: — Primer Length: 67

Sequence (5' to 3'): PTT TCT GGA TGC CGA CAG CGG AGC AAG TCC AAC GGT GGA GGC AGC GAC AGA GAT GCC GTG CCG TCT GC

Molecular Weight µg/µmole:	21897.4	µg per OD:	29.8
Millimolar Extinction Coefficient:	732.9	nmoles per OD:	1.3
Purity	**Desalted**	**OD's**	10.7
Tm (1 M Na+)	97	**µg's***	319
Tm (50 mM Na+)	78	**nmoles**	14
% GC	62	**Coupling Eff.**	99%

Notes:

UBI 1A 57 nmoles 570 µl → 100 pmol/µl

14 nmoles 140 µl → 100 pmol/µl

EXHIBIT C

* See Note about Quantities in Supporting Information.

LIFE TECHNOLOGIES

GIBCO BRL Custom Primers
Certificate of Analysis

Primer 1:

Primer Name: UBI HSPA VER.2A — Primer Number: D0373B07 (B07)
Researcher: — Primer Length: 81
Sequence (5' to 3'):P-A GAC GGC ACG GCA TCT CTG TCG CTG CCT CTG GAC CCC TCT CGA CCA CCG TTG GAC TTG CTC CGC TGT CGG CAT CCA GAA AT

Molecular Weight µg/µmole:	26105.2	µg per OD:	31.6
Millimolar Extinction Coefficient:	824.3	nmoles per OD:	1.2
Purity	Desalt	OD's	90.0
Tm (1 M Na+)	98	µg's*	2850
Tm (50 mM Na+)	77	nmoles	108
% GC	61	Coupling Eff.	98%

Notes: 10 μl → 100 pmol/μl

Primer 2:

Primer Name: UBI HSPB VER.2B — Primer Number: D0373B08 (B08)
Researcher: — Primer Length: 82
Sequence (5' to 3'):P-T TTC TGG ATG CCG ACA GCG GAG CAA GTC CAA CGG TGG TCG AGA GGG GTC CAG AGG CAG CGA CAG AGA TGC CGT GCC GTC TGC

Molecular Weight µg/µmole:	26872.4	µg per OD:	29.7
Millimolar Extinction Coefficient:	902.2	nmoles per OD:	1.1
Purity	Desalt	OD's	77.0
Tm (1 M Na+)	99	µg's*	2294
Tm (50 mM Na+)	78	nmoles	85
% GC	63	Coupling Eff.	98%

Notes: 850 µl → 100 pmol/µl

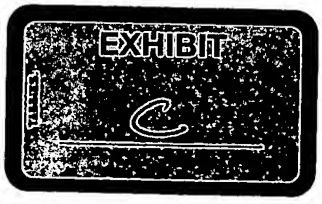
Primer 3:

Primer Name: UBI HSPA VER.3A — Primer Number: D0373B09 (B09)
Researcher: — Primer Length: 81
Sequence (5' to 3'):P-A GAC GGC ACG GCA TCT CTG TCG CTG CCT CTC GAG AGT TCC GCT CCA CCG TTG GAC TTG CTC CGC TGT CGG CAT CCA GAA AT

Molecular Weight µg/µmole:	26160.2	µg per OD:	31.5
Millimolar Extinction Coefficient:	830.8	nmoles per OD:	1.2
Purity	Desalt	OD's	88.7
Tm (1 M Na+)	98	µg's*	2783
Tm (50 mM Na+)	76	nmoles	106
% GC	60	Coupling Eff.	98%

Notes: 1060 µl → 100 pmol/µl

* -See Note about Quantities in Supporting Information.



LIFE TECHNOLOGIES™

GIBCO BRL Custom Primers
Certificate of Analysis

Primer 4:

Primer Name: UBI HSPB VER.3B — Primer Number: D0373B10 (B10)

Researcher: — Primer Length: 82

Sequence (5' to 3'): P-T TTC TGG ATG CCG ACA GCG GAG CAA GTC CAA CGG TGG AGC GGA ACT CTC GAG AGG CAG CGA CAG AGA TGC CGT GCC GTC TGC

Molecular Weight µg/µmole:	26816.4	µg per OD:	29.7
Millimolar Extinction Coefficient:	901.3	nmoles per OD:	1.1
Purity	Desalt	OD's	83.2
Tm (1 M Na+)	99	µg's*	2476
Tm (50 mM Na+)	77	nmoles	92
% GC	62	Coupling Eff.	98%
Notes:			930 µl → 100 pmol/µl

Primer 5:

Primer Name: UBI HSPA VER.4A — Primer Number: D0373B11 (B11)

Researcher: — Primer Length: 86

Sequence (5' to 3'): P-A GAC GGC ACG GCA TCT CTG TCG CTG CCT CTG GAC CCC TCT CGA CTC GAG AGT TCC GCT CCA CCG TTG GAC TTG CTC CGC TGT CGG CAT CCA GAA AT

Molecular Weight µg/µmole:	30986.2	µg per OD:	31.7
Millimolar Extinction Coefficient:	976.3	nmoles per OD:	1.0
Purity	Desalt	OD's	89.3
Tm (1 M Na+)	100	µg's*	2833
Tm (50 mM Na+)	78	nmoles	91
% GC	61	Coupling Eff.	98%
Notes:			90 µl → 100 pmol/µl

Primer 6:

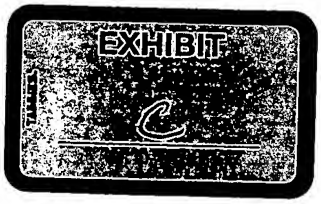
Primer Name: UBI HSPB VER.4B — Primer Number: D0373B12 (B12)

Researcher: — Primer Length: 97

Sequence (5' to 3'): P-T TTC TGG ATG CCG ACA GCG GAG CAA GTC CAA CGG TGG AGC GGA ACT CTC GAG TCG AGA CGG GTC CAG AGG CAG CGA CAG AGA TGC CGT GCC GTC TGC

Molecular Weight µg/µmole:	31791.4	µg per OD:	29.6
Millimolar Extinction Coefficient:	1070.6	nmoles per OD:	0.9
Purity	Desalt	OD's	97.1
Tm (1 M Na+)	100	µg's*	2883
Tm (50 mM Na+)	79	nmoles	90
% GC	62	Coupling Eff.	98%
Notes:			900 µl → 100 pmol/µl

* -See Note about Quantities in Supporting Information.



LIFE TECHNOLOGIES.

Do Nucleotrol preps of 5596, 5597, 4216, 4217, 4218 and 4219

Digest 5596 and 5597 w/ EcoRI as a check make sure smaller frag. is ~2kb.

Digest 4216, 4217, 4218, 4219 w/ BglI/SalI to check that the 168bp frag. is generated.

Digest 4216 w/ BcgI/XcmI to use as accepting vector for ubiquitin versions 1-4. Gel isolate on 12% agarose

Digest 5596 w/ NheI/NotI to isolate insert (~1.6 kb) 4:BABSS Gel isolate on agarose

NotI and NheI cut in different buffers
cut w/ NotI 1st @ 37°C and EtOH ppt.
cut w/ NheI

Mini-preps on BABSS:NM #s 5648 - 5665
Digest w/ EcoRI/PstI. Cut 1/8 also Run on 10% acrylamide

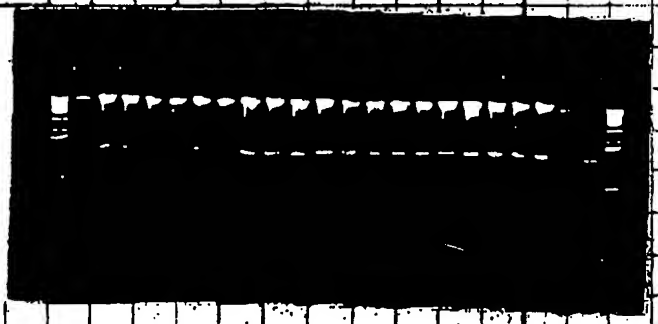
Anneal Ubiquitin versions 1, 2, 3, and 4 oligos together.
Heat to 95°C for 5 min. then stick on ice
Check these on a 10% acrylamide gel

Run precut 3770 NheI/NotI on an agarose gel to check it out. Kathleen Burns

EXHIBIT D

Check Ubi variation w/ a 113/NheI digest (first 5 of each)

1. 1Kb ladder
2-6 Ubi1 7543 - 7597
7-11 Ubi2 7561 - 7565
12-16 Ubi3 7579 - 7583
17-21 Ubi4 7597 - 7601
22 [redacted] NheI/HindIII 7062 vector
23 6939 NheI/HindIII frag
24 1Kb ladder

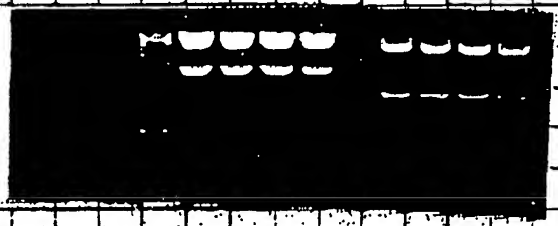


All look fine. Send these for sequencing

Sequencing data shows 7368 to be correct for Gus/PZP

Make Avidin/PZP using 7368 to start

Digest 7424 (Avidin/3770) and 7368 (Gus/PZP) with BamHI/XhoI



Gel 1 1Kb ladder
2-5 7368 (Gus/PZP) BamXho
6 Skip
7-10 7424 (Avidin) BamXho

Isolate large vector frag from Gus/PZP and the smaller insert band from Avidin

Katherine Barfoot

EXHIBIT E

Gus Assay

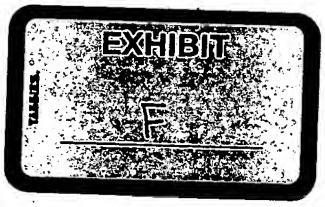
PURPOSE: TO QUANTITATE THE AMOUNT OF GUS IN CORN SEED.

MATERIALS: REACTION PLATE – COSTAR EIA/RIA
READING PLATE – NUNC FLUORONUNC POLYSORP
MU – 4 METHYLUMBELLIFERONE (SIGMA M-1508)
MUG – 4 METHYLUMBELLIFERONE β GLUCURONIDE (SIGMA M-9130)
MICROBALANCE
FLUORESCENCE MICROPLATE READER

PROCEDURE: USE PROTOCOL FOUND ON PAGE# 57 OF THIS NOTEBOOK (#58).

RESULTS: DATA FOUND BELOW. (BASED ON 20-MIN READINGS.

SAMPLE #	%TSP	SAMPLE #	%TSP
GSE 12020-4	.088	GSE 05030-1	0.087
-5	ND	-2	0.54
GSD 01120-1	ND	-3	0.61
-2	ND	-4	0.16
-3	ND	-5	0.06
-4	ND	" 0808-1	0.001
-5	ND	-2	0.002
GSE 15070-4	0.28	-3	0.007
" 05050-1	0.17	-4	ND
-2	0.015	-5	0.001
-3	0.010	" 07050-1	0.3
-4	0.174	-2	0.089
-5	0.010	-3	0.27
" 05090-1	0.043	-4	0.013
-2	0.014	-5	0.43
-3	0.001		
-4	0.004		
-5	0.024		
GSC 01010-1	0.006		
-2	0.010		
-3	0.009		
-4	0.60		
-5	~~OFF SCALE~~ ND 0.48		



Investigator: Book # 58

Chris Brook Date:

Witness: Elizabeth Wiford Date:

GUS ASSAY

SEE PURPOSE, MATERIALS, & PROCEDURE BELOW.

Purpose: To quantitate the amount of GUS in corn seed extracts.

Materials:
Reaction Pluto-Costar EIA/RIA, non-tissue culture treated 96-well flat bottom plate
Reading Pluto-Nunc Fluoronunc Polysorp 96-well black plate
MU 4-methylumbelliferone (Sigma M-1508)
MUG 4-methylumbelliferone ß-glucuronide (Sigma M-9130)
Microbalance
Fluorescence microplate reader (Molecular Devices Gemini)

Reagents:
Lysis Buffer: 50 mM sodium phosphate pH 7.0, 1 mM EDTA, 10 mM ßME.
Note: 50 mM sodium phosphate is made by mixing 97 ml of Stock A (0.2M NaH_2PO_4 (27.6 g/L)) with 153 ml of Stock B (0.2M Na_2HPO_4 (53.6 g/L) and bringing to a final volume of 1.0 L with dH_2O.
Also note that the 10 mM ßME should be added to an aliquot of the lysis buffer fresh daily, enough for that day's experiment.
Stop Buffer: 0.2 M Na_2CO_3 (21.2 g/L)
1 mM MU Standard Stock: 4.96 mg MU in 25 ml dH_2O (made fresh daily)
20 mM MUG Substrate Stock: 7 mg MUG in 1.0 ml 95% ethanol (made fresh daily).

Procedure:
Corn seed extracts should already be prepared and analyzed for total protein according to standard procedures.

In a reaction plate, equilibrate up-to-10-µg of total protein in a total volume of 100 µl lysis buffer. Generally samples can be analyzed with 1 µg total protein. Samples should be analyzed in triplicate.

Add standard curve to triplicate wells diluted as follows:

10 µl of 1 mM MU standard stock is diluted with 90 µl lysis buffer.
10 µl of this 1:10 dilution is further diluted with 90 µl lysis buffer to give a 1:100 dilution.

0 nM MU standard	100 µl lysis buffer / well
1000 nM MU standard	12.5 µl of the 1:100 dilution + 87.5 µl lysis buffer /well
10,000 nM MU standard	12.5 µl of the 1:10 dilution + 87.5 µl lysis buffer / well
100,000 nM MU standard	12.5 µl of the 1 mM MU stock + 87.5 µl lysis buffer / well

Prepare the reading plates by pipetting 175 µl of Stop buffer into all wells of the plate. You will need a separate plate for each time point required. Generally we take readings at 0, 15, 30 and 60 minutes.

Dilute the 20 mM MUG substrate stock to 5 mM with lysis buffer. Add 25 µl of 5 mM MUG to every well including both standard and sample wells and mix to start the reaction. Immediately after adding the MUG, pipette 25 µl of solution from the reaction plate into a prepared reading plate. Place the reaction plate at 37 °C until the next time point. At each subsequent time point, pipette 25 µl of solution from the reaction plate into a prepared reading plate.
Reaction is stable for several hours once it has been stopped. Note that stopping the reaction is essential for fluorescence formation.

Plates are read at 360 nm excitation wavelength and 460 nm emission wavelength.

The unknown samples are read against the standard curve in nM MU and the amount of GUS in the samples is calculated as follows:

Average nM MU for each sample (Mean Value Column)/ minutes reaction proceeded = nM MU / min * 60 min / hr = nM MU / hr. Note that if there is > 100 FU in the 0 time point reading of the average nM MU, that value must be subtracted from the average nM MU at each subsequent reading. This value is then corrected for the amount of protein added in the sample by dividing by the total protein added to give nM MU / hr / µg. This value is converted to %TSP by multiplying by 1.68×10^{-4} which is a conversion factor determined while at Pioneer.

A Quality Control sample (a known amount of GUS spiked into control corn seed extract) may be run on each assay to determine reproducibility of quantitation.

RESULTS: DATA FOUND BELOW. (10-MIN READINGS)

SAMPLE#	%TSP	SAMPLE#	%TSP	SAMPLE#	%TSP
GSG 01040-1	~~0~~ 0	GSC 01110-1	~~0.6~~ 0.06	GSC 01060-1	~~0~~ 0
-2	~~0.4~~ 0.04	-2	~~0.4~~ 0.04	-2	~~0~~ 0
-3	~~0.6~~ 0.06	-3	~~0~~ 0	-3	~~0~~ 0
-4	~~0.5~~ 0.05	-4	~~0.4~~ 0.04	-4	~~0~~ 0
-5	~~0.4~~ 0.04	-5	~~0.4~~ 0.04	-5	~~4.8~~ 0.5
GSD 02130-1	~~1.1~~ 0.1	GSC 01070-1	~~4.2~~ 0.4	GSC 01130-1	~~8.4~~ 0.8
-2	~~0.7~~ 0.07	-2	~~2.7~~ 0.3	-2	~~0.1~~ 0.01
-3	~~0.9~~ 0.1	-3	~~3.4~~ 0.3	-3	~~8.6~~ 0.9
-4	~~0~~ 0	-4	~~5.2~~ 0.5	-4	~~5.0~~ 0.5
-5	~~0.8~~ 0.1	-5	~~0.01~~ ~~0.08~~ 0.001	-5	~~0.7~~ 0.07
GSG 01020-1	~~0~~ 0	GSC 01040-1	~~0.1~~ 0.01	GSC 01110-1	~~0~~ 0
-2	~~0~~ 0	-2	~~5.1~~ 0.5	-2	~~9.2~~ 0.9
-3	~~0.12~~ 0.01	-3	~~0.3~~ 0.03	-3	~~0~~ 0
-4	~~0~~ 0	-4	~~0.3~~ 0.03	-4	~~0~~ 0
-5	~~0.2~~ 0.02	-5	~~0.04~~ 0.004	-5	~~7.6~~ 0.7
GSC 01030-1	~~0~~ 0				
-2	~~4.0~~ 0.4				
-3	~~4.2~~ 0.4				
-4	~~0.5~~ 0.05				
-5	~~7.5~~ 0.8				

Investigator: Book # 67

Investigator: Chris Brooks Date:

Witness: Elizabeth Wilson Date: